A 'litmus test' for molecular recognition using artificial membranes

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Background: Sensitive and selective molecular recognition is important throughout biology. Certain organisms and toxins use specific binding at the cell surface as a first step towards invasion. A new series of biomolecular materials, with novel optical and interfacial properties, have been designed to sense molecular recognition events. These polymers, the diacetylenic lipids, have previously been shown to undergo chromatic transitions in response to virus binding to the surface of the material.

Results: Gangliosides that specifically bind cholera toxin, heat-labile *Escherichia coli* enterotoxin and botulinum neurotoxin were incorporated into a matrix of diacetylenic lipids, 5–10% of which were derivatized with sialic acid. The lipids were self-assembled into Langmuir–Blodgett layers and polymerized with ultraviolet irradiation, yielding a polydiacetylene membrane with a characteristic blue color into which the ganglioside is non-covalently incorporated. When toxin is added, the polymerized membrane turns red. The response is specific and selective, and can be quantified by visible absorption spectrophotometry.

Conclusions: Polydiacetylenic lipid membranes offer a general 'litmus test' for molecular recognition at the surface of a membrane. A concentration of 20 ppm of protein could be detected using polymerized thin films. The speed, sensitivity and simplicity of the design offers a new and general approach towards the direct colorimetric detection of a variety of different molecules.

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Introduction

Living organisms recognize and respond selectively to numerous molecules. Certain organisms use molecular recognition at the cell surface to invade other living organisms. Engineered artificial membranes using conjugated polydiacetylene polymers can be designed to undergo distinct chromatic transitions when such a molecular recognition event occurs at the surface of a membrane (Fig. 1a). Self-assembly of these amphiphilic diacetylenic molecules occurs by the same entropic driving forces which result in the formation of biological cell membranes and vesicles [1]. Once the monomers have been assembled into an ordered array, they are polymerized by UV irradiation into a blue-colored polydiacetylene polymer [2–4]. Color changes of polydiacetylenes have been known to occur in response to a variety of environmental perturbations such as increased temperature, pH, or mechanical stress [5–8]. By incorporating bio-specific receptors into the polydiacetylene matrix, we found that molecular recognition at the surface of these materials, such as the binding of influenza virus, can similarly induce the chromatic transition [9]. The materials can also be used to form liposomes [10]. This direct colorimetric detection strategy allows molecular recognition and optical 'reporting' to occur within a single macromolecular assembly. This approach bypasses the need for secondary color development reagents, such as the enzyme-linked antibodies used in ELISA detection. The technology also allows for very rapid detection of biological molecular recognition events.

Design of supramolecular polydiacetylene assemblies

The flexibility in the chemistry and the architecture of the self-assembling materials allows investigation of a variety of design strategies for the colorimetric sensors. In this way, optimization can be readily achieved for a number of different receptor–ligand interactions. Polydiacetylene matrices have been used in two molecular architectures so far: thin polydiacetylene films coated on solid glass support (Fig. 1b, [9]), and polydiacetylene liposomes in solution (Fig. 1c, [10]). In addition to these two supramolecular architectures, two different approaches can be used to functionalize the surface of the assembly. In one case, the diacetylenic monomer lipid is directly derivatized with the appropriate receptor by synthetic coupling. This allows direct crosslinking of the 'receptor-lipid' with the surrounding polydiacetylene 'matrix'. Binding affinity or ligand stability can be controlled by suitable modification of receptor structure [9]. Here we show that covalent crosslinking between the chromophore and the receptor is not essential to generate the chromatic transition on ligand binding; a receptor molecule can be non-covalently incorporated into the polydiacetylene matrix in a manner analogous to the heterogeneous mixing of molecules in cell membranes.

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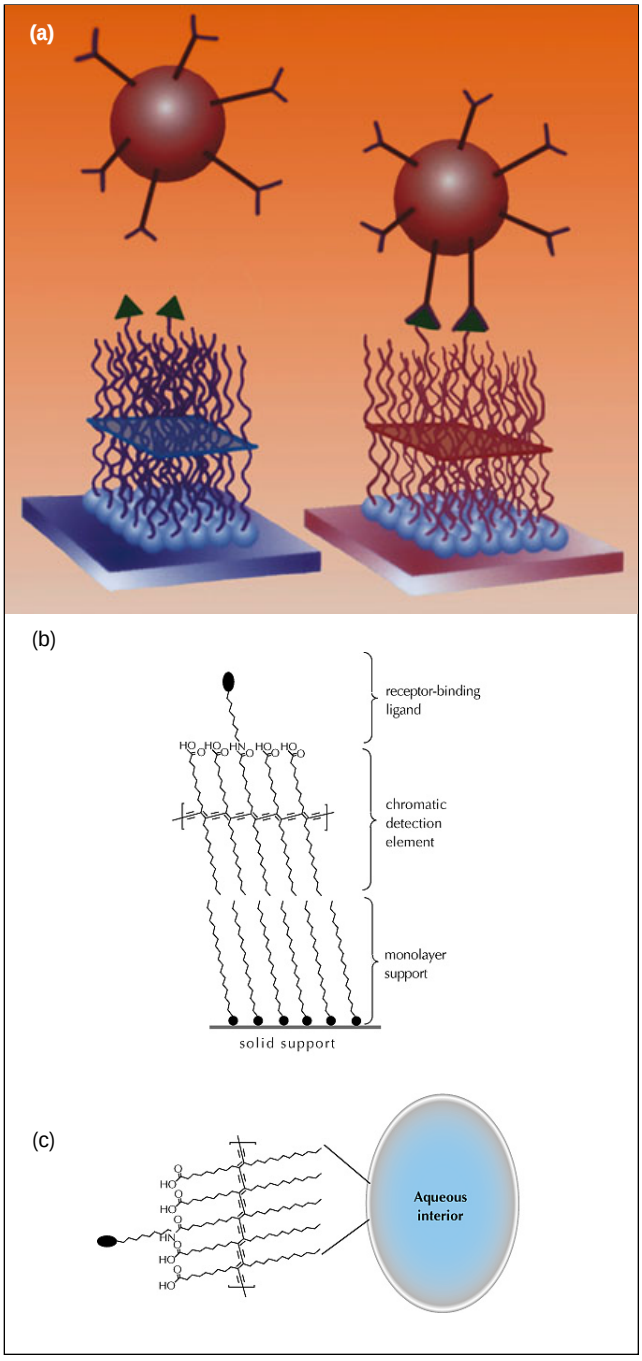

Fig. 1. Making a colorimetric biosensor. **(a)** The engineered conjugated polymers before (left, blue) and after (right, red) exposure to a multivalent analyte. Binding to a molecular recognition site induces stresses that are detected by an optical 'reporter' element, which signals the binding event by changes in the optical absorption spectrum of the polymer. The two molecular architectures used in this study are **(b)** polydiacetylene thin films on solid supports and **(c)** polydiacetylene liposomes.

Results and discussion

We describe the incorporation of several different compounds into a 'matrix' lipid, 10,12-pentacosadiynoic acid (PDA; Fig. 2, compound **1**). After covalent coupling of the C-glycoside of the carbohydrate sialic acid to PDA, this molecule (Fig. 2, compound **2**) can be crosslinked to the matrix lipid (giving the product shown in Fig. 1b). Influenza virus binds to this assembly [9,10] via the viral lectin, hemagglutinin (HA), which binds to terminal α-glycosides of sialic acid on cell-surface glycoproteins and glycolipids [11,12], thus initiating cell infection by the virus [13]. Specific, desirable properties can be built into the receptor-lipid molecule to enhance binding and stability. For example, the bifunctional sialic-acid-derivatized PDA incorporates both the sialic acid receptor for viral binding and the diacetylenic functionality in the hydrocarbon chain for polymerization. The carbon-glycoside in this compound was incorporated into the structure in such a way as to prevent hydrolysis by viral neuraminidase. The derivatized PDA can be mixed with 10,12-pentacosadiynoic acid and formed into thin films or liposomes. Optimal sialic-acid-derivatized PDA sensors are composed of 5–10 % sialic acid lipid and 90–95 % matrix lipid [14].

If the receptor of interest is already lipid-linked, it can be directly incorporated into the macromolecular assembly, avoiding potentially complex synthetic steps (Fig. 3). Gangliosides, a family of molecules that reside on the cell surface of neurons, provide a suitable system for demonstrating this approach. Gangliosides are lipid molecules that are located in the plasma membrane of cells and have a carbohydrate recognition group attached to the extracellular surface. The lipid anchors the carbohydrate in the cell membrane, and allows incorporation of gangliosides into the artificial PDA assemblies or liposomes. Two representative members of this family are the G_{M1} and G_{T1b} gangliosides (Fig. 2, compounds **4**,**5**). The G_{M1} gangliosides, present on the surface of intestinal cells, are the primary target of cholera toxin, the neurotoxin responsible for the disease cholera [15]. The G_{T1b} gangliosides are located at the neuromuscular junction, and are the primary target of botulinum neurotoxin, the neurotoxin responsible for botulism [16].

The chromatic unit of the neurotoxin sensor is composed of PDA and 'promoter' PDA. The function of the 'promoter' PDA is not fully understood, but it is essential to produce the chromatic transition in the toxin-binding experiments. We postulate that the 'promoter' PDA lowers the activation barrier of the chromatic transition. The promoter probably changes lipid packing, altering the effective conjugated length of the backbone, or it may provide a connection between the non-conjugated receptor and the conjugated backbone, enabling the neurotoxin to induce the colorimetric transition. The promoter PDA used in these investigations is a sialic-acid- or lactose-derivatized PDA lipid (Fig. 2, compounds **2**,**3**). It should be noted that, in this case, the derivatized lipid is used to modify the film's optical properties, and not as a molecular recognition site as in the case of influenza virus detection. The polydiacetylene bio-assembly containing only sialic-acid-derivatized PDA (or lactose-derivatized PDA) does not respond to the neurotoxins used in this study (data not shown), indicating that there is not sufficient interaction between the neurotoxins and the derivatized diacetylene lipid to induce the color change.

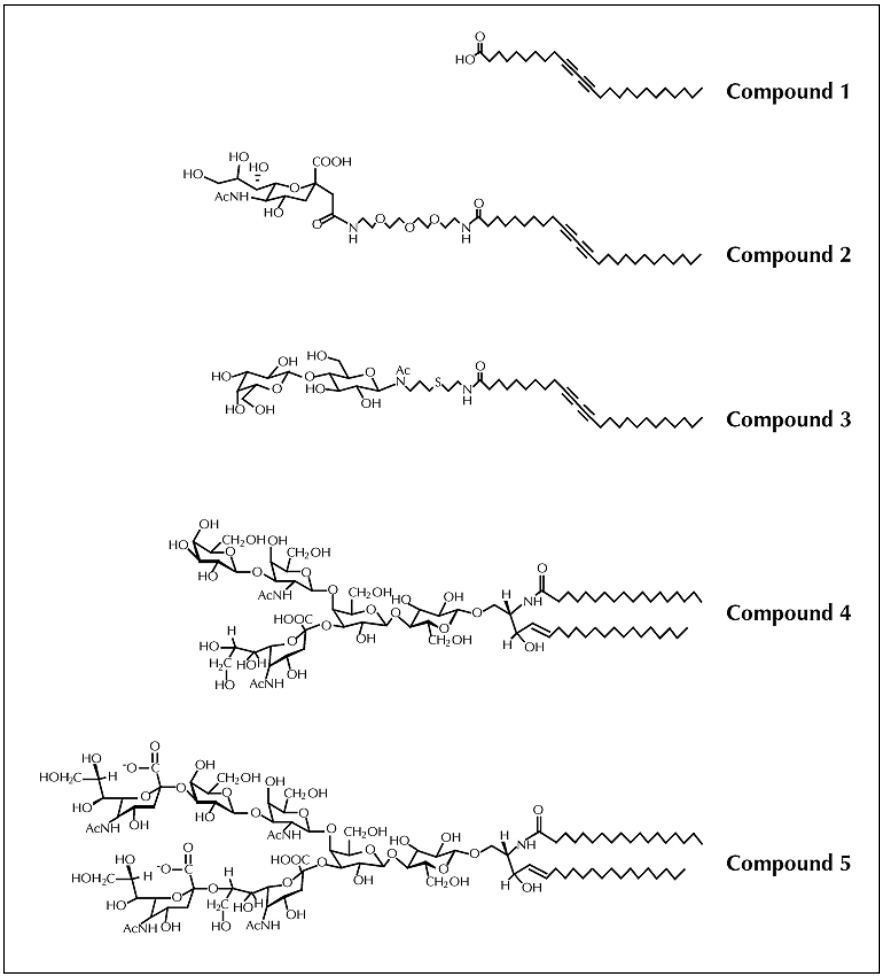

Fig. 2. Lipids and lipid-linked cell-surface moieties used in the design of colorimetric sensors of biological ligands. Compound **1** is the matrix lipid 10,12-pentacosadynoic acid (PDA). PDA derivatized with sialic acid (compound **2**) is used as a binding site for influenza virus hemagglutinin or as a 'promoter' lipid for toxin-binding studies; lactose-derivatized PDA (compound **3**) is also used as a 'promoter' lipid in assemblies incorporating the ganglioside G_{M1} (compound **4)** and G_{T1b} (compound **5**). The gangliosides occur naturally in cell membranes, and are composed of a carbohydrate head group used in molecular recognition, and a ceramide lipid chain that resides within the cell membrane.

To self-assemble the most sensitive layers to detect toxin binding, typically a mole ratio of 90 % PDA, 5 % sialic-acid-derivatized PDA and 5 % ganglioside lipid is used. If more than 5 % ganglioside is used, polymerization is reduced to such an extent that the films are of poor optical quality, due to steric hindrance of the solid-state polymerization. For films containing the lactose promoter lipid, it was found that too high a concentration of lactose-derivatized PDA (> 5 %) also led to unstable films that turned red upon exposure to buffer solutions. In optimal conditions, the self-assembled monolayer of 2 % lactose-derivatized PDA, 5 % ganglioside and 93 % PDA resulted in a blue to red color change when the film was incubated specifically with cholera toxin.

Colorimetric detection of viruses and neurotoxins

The thin-film toxin biosensors obtained in this study exhibit the characteristic blue color due to the presence of conjugated, planar, rigid polydiacetylene chains in the molecular assembly (Figs 4a). This can be compared with the color of the liposome sensor for influenza virus (Fig. 5a, [10]. The optical properties of these sensors can be quantified by visible absorption spectroscopy (Figs 4b,5b). For example, the blue colored film has an absorption maximum of ~ 630 nm and a weaker absorption at ~ 550 nm. After incubation with the target analyte, a dramatic change in the visible spectrum occurs. The maximum at ~ 550 nm increases with a concurrent decrease in the maximum at ~ 630 nm, and the film or liposome suspension appears red. The color change can be quantified by calculation of the colorimetric response (CR) by measuring the relative change in the percentage of the intensity at ~ 630 nm relative to the intensity at ~ 550 nm [9].

To determine the sensitivity of the biosensors to target analyte, the response (CR) of the sensor as a function of analyte concentration was determined (Fig. 4c). In agreement with previous results, the CR is directly proportional to the quantity of target analyte [9,10]. For the G_{M1}-containing biosensor, the colorimetric response to cholera toxin rises steeply at low toxin concentration, then levels out at higher concentration, indicating that surface binding sites are saturated (Fig. 4c). The low detection limit corresponds to a sensitivity of $\sim 1 \times 10^{-10}$ M. The absolute sensitivity of the lactose-derivatized PDA doped film is slightly lower than that of films containing the sialic acid promoter lipid, due to the higher background level in the presence of buffer only (CR = 7 % for lactose-PDA; CR = 5 % for sialic acid-PDA). Similar results are seen using the G_{T1b} ganglioside biosensor and botulinum neurotoxin, and using the G_{M1} ganglioside biosensor to detect *E. coli* enterotoxin (data not shown).

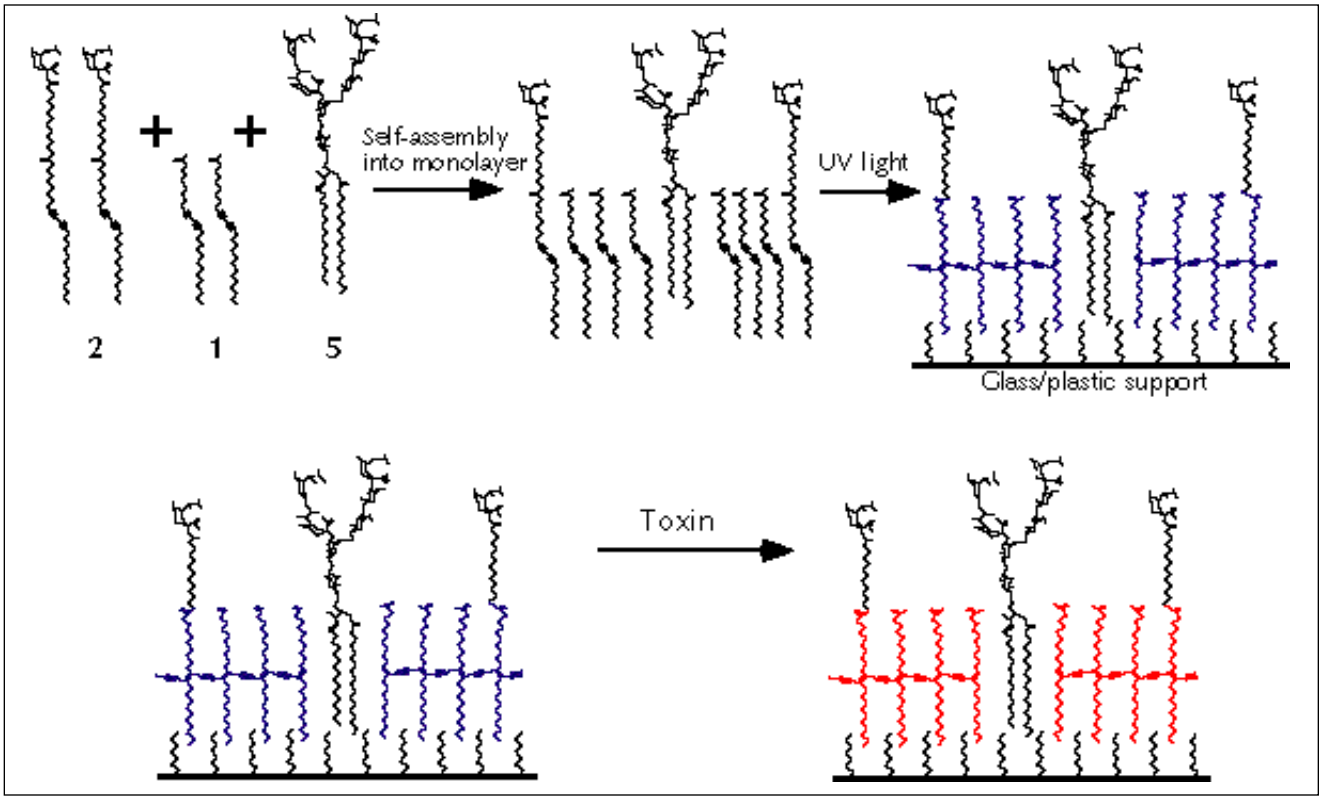

Fig. 3. Construction of heterogeneously polymerized thin-film assemblies for the detection of toxins. Films were assembled from a starting solution of an organic mixed solvent (chloroform:methanol 2:1) containing 2–5 % sialic-acid- or lactose-derivatized PDA (compound **2** or **3**), 90–93 % PDA (compound **1**) and 5 % ganglioside G_{M1} or G_{T1b} (compound **4** or **5**) as detailed in Materials and methods. After polymerization, the film displays a steady blue color, but upon exposure to toxin the film turns red.

To demonstrate that the incorporation of G_{M1} into the biosensor assembly did not compromise the G_{M1}–cholera toxin interaction, the supramolecular array was self-assembled onto a gold chip, and the interaction was measured by surface plasmon resonance using a Biacore 2000 instrument. The binding affinity (K_a) of cholera toxin to the G_{M1} biosensor was determined to be 3×10^{10} M^{-1} [17], in agreement with the published values observed *in vivo* [15]. This suggests that G_{M1} incorporated into the artificial membrane behaves similarly to G_{M1} on the surface of living cells.

To evaluate the selectivity of the sensor material, a series of experiments were carried out to confirm that the functionalized polydiacetylene assemblies are specific to the biological target. For example, *Escherichia coli* cell lysate, bovine serum albumin, pertussis toxin, diphtheria toxin, and various buffers at different salt and pH conditions produce a background CR of ~5 % (Fig. 6); the highest background was seen using bovine serum albumin (BSA). These results define the level of non-specific adhesion, and therefore, the minimum detection limit. Low levels of toxin or virus molecules yield CRs significantly above the background level (Fig. 6). No cross reactivity was observed between the neurotoxin and virus sensors. The majority of the colorimetric response occurs immediately upon exposure to the target analyte. After one second, 75 % of the total CR to target analyte is observed to occur (Fig. 6a).

A much higher concentration of polymerized material can be achieved with liposome solutions compared to monolayer assemblies (Fig. 1), due to their greater cross-sectional density. Liposomes have the advantage of making the color change more visually striking and increasing the colorimetric response (compare Figs 4,5). The liposomes are prepared from the same starting monomers as would be used for thin film assembly, typically using a probe sonication method. The optical absorption properties of the liposomes can be controlled to a certain extent by the polymerization time (Fig. 5b). Typically, blue liposomes turn pink, while purple liposomes turn orange upon addition of target analyte. As noted with the neurotoxin sensors, and as previously shown [10], the colorimetric response increases with increasing amounts of analyte. No color change could be detected if pure PBS buffer (phosphate buffered saline) or a solution of bovine serum albumin (BSA) in PBS buffer (1 mg ml^{-1}) was added to the virus sensor system (Fig. 6). The amount of virus that can be detected above the background is 8 hemagglutination units (HAU; one HAU is defined as the highest dilution of stock virus that completely agglutinates a standard erythrocyte suspension [18], and 8 HAU corresponds to ~ 8×10^7 virus particles [19]). As illustrated in Figure 6, the liposome sensor produces a much higher response to virus binding than the thin film sensor does for toxin binding. Typically, the liposome sensors have CR values of ~ 70 %, relative to typical thin-film CR values for toxins of ~ 30 %.

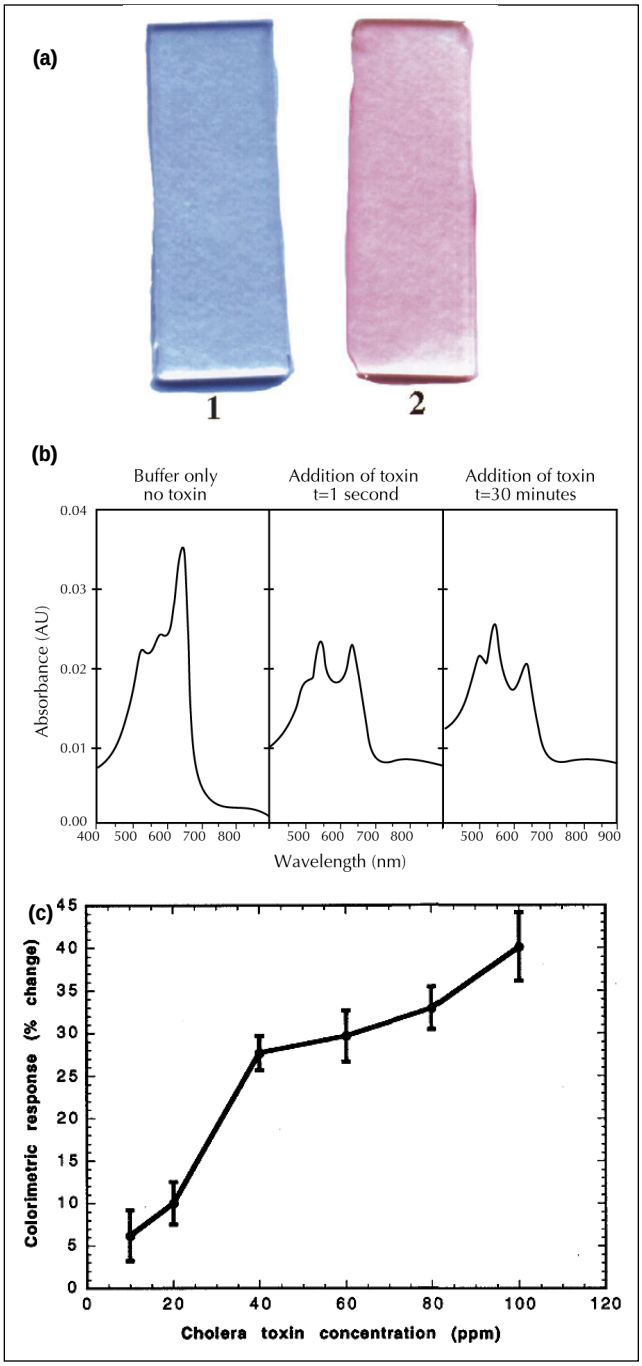

Fig. 4. Colorimetric, spectrophotometric, and titration data for cholera toxin biosensor with the G_{M1} ganglioside. **(a)** The untreated G_{M1} sensor placed on silanized glass cover slides (left) is blue, but minutes after exposure to 40 ppm cholera toxin is red (right). **(b)** UV–VIS spectra of 5 % G_{M1}, 5 % sialic acid–PDA, 90 % PDA sensor before (left) and after (middle, immediately after; right, 30 min after) exposure to 40 ppm cholera toxin. **(c)** Plot of the colorimetric response of the biosensor used in (b) as a function of cholera toxin concentration in ppm. Each point in the graph is the average value of four measurements, with the standard deviation as the error.

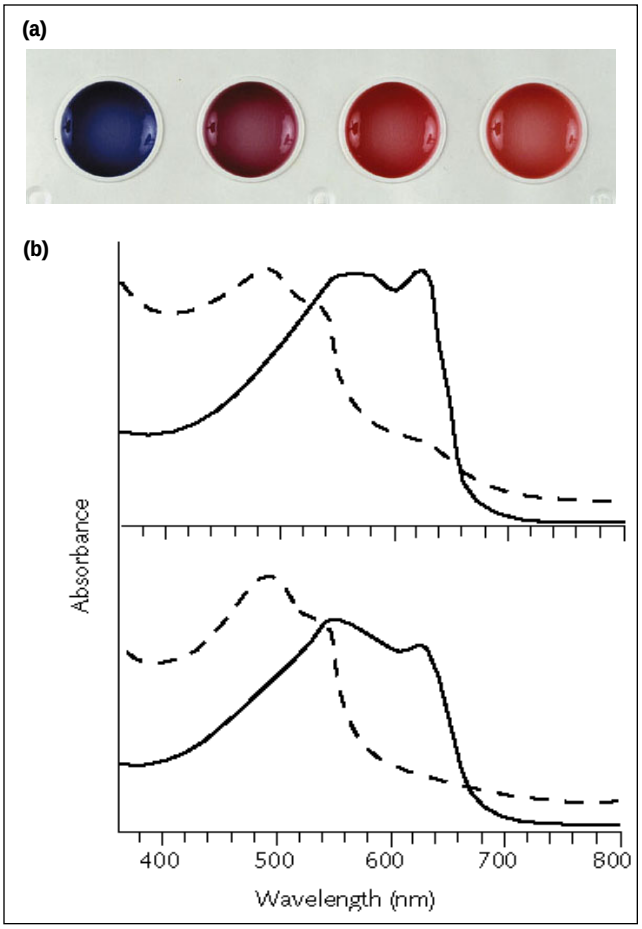

Fig. 5. Colorimetric detection of influenza virus using polymerized liposomes containing sialic acid. **(a)** Photograph of liposomes to which have been added increasing amounts (from left to right) of influenza virus. Liposomes were 5 % sialic acid–PDA and 95 % tricosadiynoic acid. To each well was added the following amounts of influenza virus (left to right): 0 HAU, 8 HAU, 16 HAU, 32 HAU. **(b)** Visible absorption spectra before (solid line) and after (dashed line) incubation with influenza virus starting with either a blue liposome solution (top, 8 min UV treatment) or purple liposome solution (bottom, 24 min UV treatment). The concentration of the liposome solutions in PBS buffer was 0.13 mM. The solution was incubated with 60 HAU of influenza virus for 1 h.

The specific nature of the interaction between the influenza virus and the sialic acid-PDA liposomes was confirmed by a competitive inhibition experiment (Fig.7). In the presence of α-O-methyl neuraminic acid, a known inhibitor of influenza virus hemagglutinin, no color change is observed.Therefore, the color transitions seem to be induced by specific binding of biological targets to the membrane-like surface.These results suggest that this methodology could be useful for detecting a wide variety of potential antiviral or antitoxin drugs in a high-throughput screening format.

Mechanism of action

The blue to red transition arises from a reduction in the length of the conjugated lipid backbone, most likely due to the loss of backbone planarity.The reduced conjugation results in absorption of shorter wavelengths, so red light is reflected. However, the precise molecular events leading up to the non-planar backbone geometry are not fully understood. The mechanism probably varies for different methods of inducing the transition (temperature, stress,

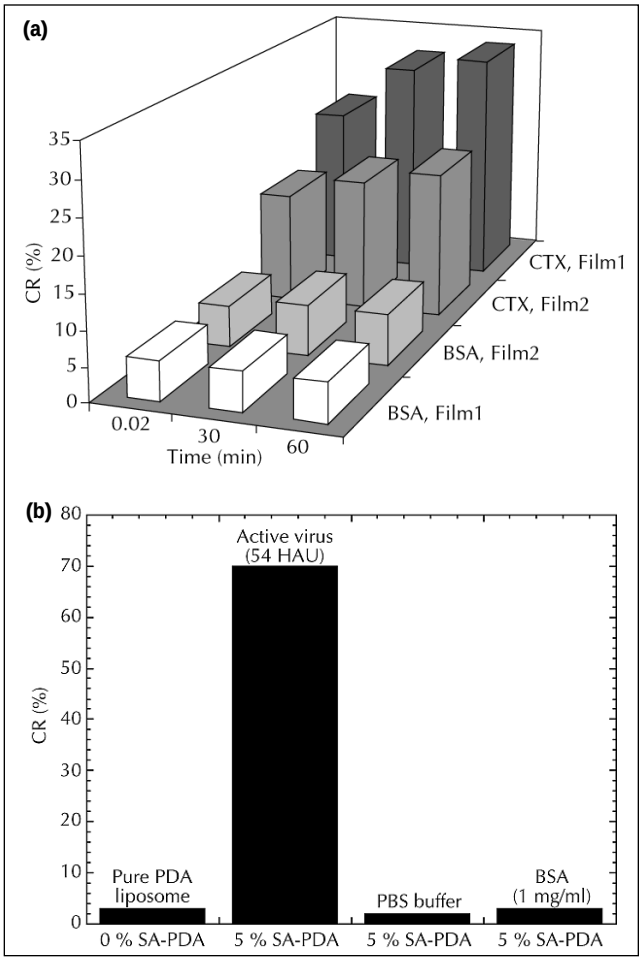

Fig. 6. Control experiments to determine the selectivity of sensors. **(a)** Either film 1 (5 % G_{M1}, 5 % sialic acid–PDA, 90 % PDA) or film 2 (5 % G_{M1}, 2 % lactose–PDA, 93 % PDA) were used to measure the colorimetric response to the addition of bovine serum albumin (BSA, 40 ppm) or cholera toxin (CTX, 40 ppm final) at time 1 s (0.02 min), 30 min, and 60 min. The colorimetric response did not change significantly after 60 min. Other neurotoxins and BSA were used to determine the selectivity of the toxin sensor, but only BSA is illustrated here, since it induced the largest response out of all of the negative controls. **(b)** Virus controls with liposomes. No color change could be observed after the addition of 54 HAU of virus if the sialic acid lipid was removed from the molecular assembly (column 1). Column 2 shows the response of liposomes that contain the sialic acid ligand to 54H AU of virus. The background CR seen in response to PBS buffer only, and 1 mgm l^{-1} bovine serum albumin are also shown.

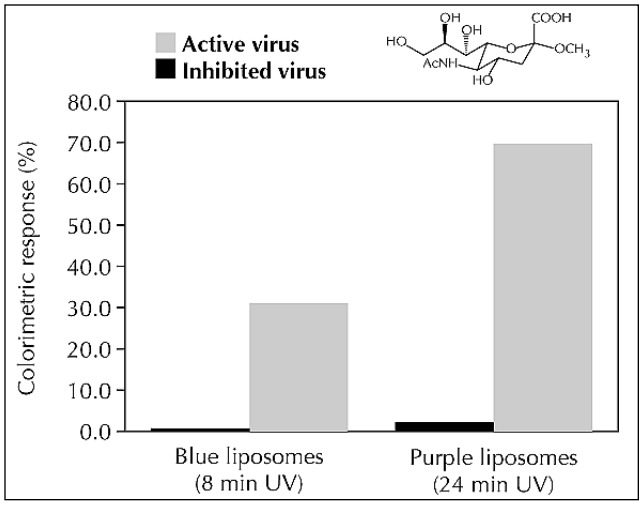

Fig. 7. Incubation of a liposome solution (10 % sialic acid–PDA) with 54 HAU of influenza virus yields a CR of 31 % for blue, and 70 % for purple, liposomes (light shaded bars). Performing the same experiment in the presence of the inhibitor α-O-methyl neuraminic acid (shown above) results in minimal color change (dark bars), illustrating the inability of virus to bind to the liposome structures.

pH, affinity). It has been postulated that the blue to red color transition arises from changes in the conjugation lengths and bond angles in the polydiacetylene backbone [2,20]. Theoretical and experimental evidence suggests that small conformational changes in the polymer side chains affect the electronic properties of the polymer backbone [21,22]. Theoretical calculations predict that as little as a 5° rotation about the C–C backbone bond could account for the observed changes in the electronic structure of the backbone [22]. Since the phenomenon of inducing the colorimetric transition by molecular recognition has only been observed very recently, investigations into the optical properties of these conjugated polymers are only now beginning.

A number of mechanisms for the color change are plausible. In the case of viral binding, a disruptive effect may be induced by the multivalent nature of the large virus particle, producing large scale changes in the conformation of the polymer side chains upon virus binding to many different binding sites. This is analogous to previous work showing that the optical properties of polydiacetylenes are altered in response to strain [23,24]. It was recently shown by FTIR that rotations about the C–C bond β to the polymer backbone is sufficient to induce the color transition [25]. These results are in agreement with previous NMR studies of thermochromism [26]. Therefore, conformational changes in the polymer's alkyl side chains appear to be a sufficient condition for the chromatic transition. It is also possible that certain protein and lipid domains of the virus insert into the membrane, inducing a conformational change of the inserted domain while inside the membrane [27]. The insertion event could disrupt the conjugated backbone, and so induce the colorimetric transition. The insertion process may also play a role in the colorimetric response induced by neurotoxins. Certain neurotoxins contain a translocation domain in addition to a binding domain and catalytic domain. For example, botulinum neurotoxin will bind to its cell surface receptor, G_{T1b}, with its binding domain, followed by insertion of the translocation and catalytic domain into the cell through the cell membrane [28], in a process reminiscent of the first stages of viral entry. This insertion may serve to disrupt the lateral interactions in the headgroup region of the film and induce the color transition. Since some neurotoxins are multimeric (such as the pentavalent cholera toxin), a multivalent model is also possible for inducing the colorimetric response.

What is the role of the 'promoter' molecule (either sialic-acid- or lactose-derivatized PDA) in the ternary film

assembly? It appears that the promoter does not function in the molecular recognition event itself (as if the receptor is omitted from the assembly there is no colorimetric change), but may serve to decrease the activation barrier for the blue to red transition. The study of a wide variety of promoter lipids should resolve this question. One possibility is that the bulky sugar headgroup in the promoter is subject to various solvent interactions at the matrix surface, destabilizing the structure of the blue film and thus allowing the relatively small perturbations provided by the toxin to complete the colorimetric transition. Since the gangliosides are not covalently connected to the PDA backbone, it also seems possible that the steric effects induced by the molecular recognition event may interfere with the headgroups of the promoter lipid, thus propagating the changes resulting from recognition to the chromatic unit of the sensor.

Significance

We have described a general technology for detecting molecular recognition events between ligands and multivalent or invasive target analytes using a simple and direct colorimetric assay. The simplicity of the technology may make it particularly useful for detecting toxic agents, ranging from single proteins to whole organisms. Although the mechanism of detection is not fully understood, the presence of 'promoter' molecules is clearly important in some systems. Changing the identity of the 'promoter' molecules may allow the detection of ligand–receptor binding events that are not multivalent, and do not involve membrane invasion.

This approach may also have applications in the area of ligand screening. As well as the possibility that the ligand binding to a receptor molecule could be made to induce the colorimetric response, the colorimetric response could also be inhibited by drug binding to the target molecule. As more discoveries are made regarding biological recognition and mechanisms of signal transduction, it will be possible to develop new generations of materials with specialized functions to mimic natural systems more closely. Use of systems such as these to mimic cell membranes and to detect events occurring within the membrane should allow an expansion of knowledge about viral fusion and toxin translocation.

Materials and methods

Construction of thin film assemblies for the detection of toxins

To construct heterogeneously polymerized thin film assemblies to detect toxins, an organic mixed solvent (chloroform:methanol, 2:1) containing 2–5 % sialic-acid- or lactose-derivatized PDA (compound **2** or **3**), 90–93 % PDA (compound **1**), and 5 % G_{M1} or G_{T1b} (compound **4** or **5**) was dispersed onto the surface of a Langmuir–Blodgett (LB) trough from KSV (Helsinki, Finland) containing 1×10^{-3} M Cd^{2+} as the subphase. The substances were equilibrated at room temperature for 30–60 min to allow organic solvent to evaporate, compressed to the solid-analogous phase, and transferred to the glass slides precoated with octadecyltrichlorosilane through vertical dipping at the speed of 5 mm min^{-1}. The resulting LB films were polymerized 1 min per side with a UVP mineralight (Fisher). After polymerization, the film displays a steady blue color.

Colorimetric and spectrophotometric detection of cholera toxin binding

Toxin binding studies were carried out with the biosensor containing G_{M1} ganglioside in a buffer consisting of 50 mM Tris, 200 mM NaCl, 1 mM EDTA and 3 mM NaN_3, pH 7.4. The films were first incubated in the buffer solution; films remaining in the buffer served as the background reference when toxins were added to generate a color change. The color change could be detected visually, and was quantified using a Perkin-Elmer Lambda 11 UV-VIS spectrometer. The response time was studied by monitoring the spectra of the biosensor immediately after the film was exposed to a solution containing 40 ppm cholera toxin. Three time points were taken to measure the colorimetric response: 1 s (0.02 min), 30 min and 60 min. The colorimetric response did not change significantly between 1 h and 24 h. The dynamic response range of the cholera toxin biosensor was determined with the film containing 5 % G_{M1} ganglioside, 5 % sialic acid–PDA and 90 % PDA as a function of cholera toxin concentration in ppm.

Construction of sialic-acid-derivatized diacetylene liposomes for detection of influenza virus

Lipids (compounds **1** and **2**) were mixed in chloroform in a test tube, and the organic solvent was evaporated to yield a thin film of the lipids on the glass. An appropriate amount of deionized water was added to give a total lipid concentration of 1 mM. The sample was heated to 80 °C, and sonicated for 15 min. The warm solution was filtered through a 0.8 mm nylon filter to remove undispersed lipid, and then cooled to 4 °C. Prior to polymerization, the liposome solution was purged with N_2 for 5 min after warming to ambient temperature. The polymerization was achieved by irradiating the solution with a UVP lamp (~ 254 nm) at a distance of 3 cm with varying irradiation times.

Colorimetric detection of influenza virus

In the wells of an ELISA plate, 200 μl of phosphate buffered saline (PBS, pH 7.4) was mixed with 30 μl of the liposome solution (5 % compound **2**, 95 % compound **1**). The reaction was started by adding the appropriate amount of influenza virus (in most cases, 30 μl), PBS as a reference, or BSA in PBS (1 mg ml^{-1}). UV spectra were recorded after various times until the color of the liposome/virus solution was unchanged.

Control experiments to determine the selectivity of toxin sensors

Cholera toxin (CTX), bovine serum albumin (BSA) and a variety of other neurotoxins (including pertussis toxin and diphtheria toxin) were used to determine the selectivity of the toxin sensor (all toxins used at 40 ppm). Only BSA is illustrated as a negative control in Figure 6, since BSA induced the largest response of all of the negative controls. The response of both Langmuir–Blodgett films and liposomes were studied, with either sialic acid-PDA or lactose-PDA. No color change could be observed if the sialic-acid lipid or lactose lipid was removed from the molecular assembly.

Inhibitor control experiments to block virus binding

In a microtiter plate well, 20 μl of virus solution (54 HAU), 100 μl of PBS buffer and 50 μl of inhibitor in PBS (100 mM

α-O-methyl neuraminic acid) were mixed and preincubated for 1 h at room temperature. In two other wells, 150 μl of PBS buffer was mixed either with 20 μl of active virus or 20 μl of buffer. To start the reaction, 20 μl of the liposome solution (10 % sialic acid-PDA) was added to each well. Again, the changes in UV spectra were monitored over time.

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